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(54) Title: **δ-ERYTHROID KRÜPPEL-LIKE FACTORS AND METHODS OF USE**

(57) Abstract

The invention features δ-erythroid krüppel-like factors (δ-EKLFs), and methods of using nucleic acids encoding δ-EKLFs to increase δ-globin gene expression in a cell.

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δ-ERYTHROID KRÜPPEL-LIKE FACTORS AND METHODS OF USE

Background of the Invention

This invention relates to gene therapy methods for treating
5 hemoglobinopathies.

The major form of adult human hemoglobin, HbA, consists of a tetramer
of two α-globin chains and two β-globin chains ($\alpha_2\beta_2$). Hemoglobinopathies,
such as sickle cell anemia and β^o-thalassemia, are caused by a failure to
produce normal levels of β-globin. Hemoglobin A₂ (HbA₂), which consists of a
10 tetramer of two α-globin chains and two δ-globin chains ($\alpha_2\delta_2$), is produced in
low amounts in most sickle cell patients and in normal adults (2-3% of total
hemoglobin) (Steinberg et al., Blood 78:2165, 1991). HbA₂ is a potent
inhibitor of the sickle hemoglobin (HbS; $\alpha_2\beta^s_2$) polymerization characteristic of
sickle cell anemia (Nagel et al., Proc. Natl. Acad. Sci. USA 76:670, 1979), and
15 shares some functional activity with HbA.

Summary of the Invention

We have shown that modification of the δ-globin gene promoter to
include a binding site for the erythroid krüppel-like factor (EKLF) polypeptide
that binds to the β-globin gene promoter (hereinafter "β-EKLF") results in
increased expression from the δ-globin promoter. A modified β-EKLF
20 (hereinafter "δ-EKLF") that binds to the wild type δ-globin gene promoter can
thus be used to induce δ-globin expression.

Accordingly, in one aspect, the invention features a method of inducing
δ-globin gene expression in a cell, such as an erythrocyte precursor cell (e.g.,
25 an erythrocyte burst-forming cell (BFC-E) or an erythrocyte colony-forming
cell (CFC-E)) or an erythrocyte. In this method, a nucleic acid encoding a δ-

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EKLF polypeptide is introduced into the cell, or a precursor of the cell. Cells into which the nucleic acid can be introduced include erythrocyte precursors, such as BFC-E and CFC-E. Preferably, the nucleic acid is introduced into a pluripotent hematopoietic stem cell, which is capable of self renewal, and thus, 5 in the context of gene therapy methods (see below), minimizes the number of treatments required.

The invention also includes the use of a nucleic acid encoding a δ-EKLF polypeptide in the preparation of a medicament for inducing δ-globin gene expression in a cell.

10 The cell into which the nucleic acid is introduced can be in a mammal or can be a cell that has been removed from a mammal for introduction of the nucleic acid, after which the cell, or progeny thereof, are introduced into a mammal. Typically, this method is carried out for a human patient. In one particular example, the nucleic acid can be introduced into a hematopoietic 15 stem cell that has been obtained from a patient. Induction of δ-globin gene expression by this method can be used in gene therapy methods for the treatment of hemoglobinopathies, such as sickle cell anemia and β⁰-thalassemia.

20 The invention also features δ-EKLF polypeptides, which are identical to β-EKLF, except that they contain one or more modifications that permit them to bind to double stranded DNA containing the sequence 5'-TGA AAC CCT-3' or the sequence 5'-CTA ATG AAA-3'. The modifications that generate δ-EKLF polypeptides are generally in a DNA-binding amino acid of a zinc finger of the β-EKLF polypeptide. For example, modifications can be made in any of 25 amino acids -1 and/or 2-6 in any of the three β-EKLF zinc fingers (see below and Fig. 2).

Preferably, the δ-EKLF polypeptide of the invention is in a substantially pure preparation. By "substantially pure" is meant a preparation that is at least

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60% by weight (dry weight) a δ-EKLF polypeptide. Preferably the preparation is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight a δ-EKLF polypeptide. Purity can be measured by any appropriate method, e.g., column chromatography, polyacrylamide gel electrophoresis, or

5 HPLC analysis.

Also featured in the invention is a nucleic acid, such as a nucleic acid containing deoxyribonucleotides (DNA), ribonucleotides (RNA), or combinations or modifications thereof, encoding δ-EKLF polypeptides.

Preferably, the nucleic acid is in the form of purified DNA. By "purified" 10 DNA" is meant DNA that is not immediately contiguous with both of the coding sequences with which it is immediately contiguous (one on the 5' end and one on the 3' end) in the naturally occurring genome of the organism from which it is derived. The term thus includes, for example, a recombinant DNA molecule that is incorporated into a vector, into an autonomously replicating 15 plasmid or virus, or into the genomic DNA of a prokaryote or eukaryote, or which exists as a separate molecule (e.g., a cDNA or a genomic DNA fragment produced by PCR or restriction endonuclease treatment) independent of other sequences. It also includes a recombinant DNA molecule that is part of a hybrid gene encoding additional polypeptide sequence.

20 The invention also includes cells, such as hematopoietic stem cells or erythrocyte precursor cells, e.g., BFC-E or CFC-E, that contain nucleic acids encoding δ-EKLF polypeptides.

Vectors containing nucleic acids encoding δ-EKLF polypeptides are 25 also included in the invention. The vectors of the invention include those that can be used in gene therapy methods for treating hemoglobinopathies, such as sickle cell anemia and β⁰-thalassemia. For example, adeno-associated viral (AAV) vectors and retroviral vectors (e.g., moloney murine leukemia viral vectors) can be used in the invention. Vectors that can be used for amplifying

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nucleic acids encoding δ-EKLF polypeptides in bacteria are also included in the invention. Preferably, the nucleic acids encoding δ-EKLF polypeptides are operably linked to a promoter, for example, the β-globin promoter, or a non-tissue specific promoter, such as the Cytomegalovirus promoter. By "operably linked" is meant that a gene and a regulatory sequence(s), such as a promoter, are connected in such a way as to permit gene expression when the appropriate molecules (e.g., transcriptional activator proteins or proteins which include transcriptional activation domains) are bound to the regulatory sequence(s).

The invention also features methods for identifying δ-EKLF polypeptides. In these methods, a nucleic acid containing either the sequence 5'-TGA AAC CCT-3' or the sequence 5'-CTA ATG AAA-3' is contacted with a candidate polypeptide that has been modified such that it differs from wild type β-EKLF by at least one amino acid, for example, an amino acid in a β-EKLF zinc finger that binds DNA. δ-EKLF polypeptides are then identified by their ability to bind to this nucleotide sequence.

Expression of therapeutic levels of gene products in gene therapy methods is often difficult to achieve. Transcription factors are generally required in lower quantities than other types of therapeutic gene products. Thus, an advantage of the present invention is that it provides a gene therapy method involving expression of a transcription factor, δ-EKLF.

Other features and advantages of the invention will be apparent from the following detailed description, the drawings, and the claims.

Brief Description of the Drawings

Fig. 1A is a schematic representation of the β-globin locus on human chromosome 11.

Fig. 1B is a schematic representation of a comparison of portions of the human δ- and β-globin promoters, and modifications of the δ-globin promoter

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to contain β -EKLF binding sites. The β - and δ -globin promoters were aligned using the GAP program of the GCG/Wisconsin package, and the regions of interest are shown. The consensus β -EKLF binding site in the β -globin promoter (CCACACCCT) is located at nucleotide positions -85 to -93 from the transcription start site and this sequence is underlined. The wild type δ -globin promoter region has a partial EKLF binding site (ACCCT) at nucleotide positions -81 to -85. To create the modified δ^{CAC-1} promoter, site-directed mutagenesis was used to convert the partial β -EKLF binding site in the δ -globin promoter to a consensus β -EKLF binding site. The δ^{CAC-2} promoter contains the β -globin EKLF binding site at nucleotide positions -85 to -93 of the δ -globin promoter, which is at the same distance from the transcription start site as the β -globin promoter EKLF binding site.

Fig. 2 is a schematic representation of the amino acid sequences of β -EKLF zinc fingers 1-3. Amino acids that can be randomized for selecting δ -globin promoter binding variants include those that are boxed.

Fig. 3A is a schematic representation of δ -globin promoter/reporter plasmids. The 1.5 kb *Kpn*I-*Bgl*II LCR HS2 fragment was linked to the -265 to +48 wild type β -, δ -, and modified δ -globin promoters described in the above description of Fig. 1B. These promoters were used to drive luciferase reporter gene expression in transiently transfected MEL cells. Luciferase activity was normalized to β -galactosidase expression from an internal control CMV- β -galactosidase plasmid.

Fig. 3B is a graph showing the results of transient expression of HS2 δ -promoter/Luciferase plasmids in mouse erythroleukemia (MEL) cells. HS2 β /Luc expression was normalized to 100%. Inclusion of the consensus β -EKLF binding site in the δ^{CAC-1} and δ^{CAC-2} promoters increased HS2 δ /Luc expression from 10% to 56% and 99% of HS2 β /Luc, respectively.

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Fig. 4A is a schematic representation of HS2 δ - β constructs. The parental HS2 δ - β construct contained a 1.9 kb *Kpn*I-*Pvu*II HS2 fragment, a 3.0 kb δ -globin gene fragment beginning at nucleotide -400 from the transcription start site, and a 2.1 kb β -globin gene fragment beginning at base -815 from the transcription start site. The corresponding δ^{CAC-1} and δ^{CAC-2} modifications were inserted to create HS2 δ^{CAC-1} - β and HS2 δ^{CAC-2} - β . These constructs were transfected into MEL cells with a pgk-neo selectable marker and G418 resistant populations were selected. The cells were induced to differentiate with DMSO, and RNA was extracted for analysis.

Fig. 4B is a photograph of denaturing gel analysis of the expression of modified δ -globin genes in MEL cells stably transfected with HS2 δ - β constructs. The relative levels of δ - and β -globin mRNAs were determined by the Single Nucleotide Primer Extension (SNuPE) assay (Szabo et al., Genes Dev. 9:1857, 1995) and quantitated by phosphorimage analysis. Inclusion of the consensus β -EKLF binding site in the δ^{CAC-1} and δ^{CAC-2} globin genes increased δ -globin mRNA levels from 3.6% to 24.7% and 34.8% of total $\delta+\beta$ mRNA, respectively.

Fig. 5A is a schematic representation of the reporter plasmid HS2 δ^{GAL4} - β . This construct contains a consensus GAL4 binding site (expanded), replacing δ -globin promoter nucleotides -80 to -99.

Fig. 5B is a schematic representation of pClneo effector constructs expressing either the GAL4 DNA binding domain (amino acids 1-147) alone or GAL4₍₁₋₁₄₇₎ fused to the β -EKLF activation domain (amino acids 2-275).

Fig. 5C is a photograph of denaturing gel analysis showing that GAL4/ β -EKLF activates δ -globin gene expression in an HS2 δ^{GAL4} - β construct. HS2 δ^{GAL4} - β was stably transfected into MEL cells with either pClneo-GAL4₍₁₋₁₄₇₎ or pClneo-GAL4₍₁₋₁₄₇₎/ β -EKLF, and relative transcript levels were determined by the SNuPE assay. The level of human $\delta/\delta+$ was 4.4% in cells

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containing HS2 δ - β , 13.3% in cells containing HS2 δ^{GAL4} - β plus pCIneo-GAL4₍₁₋₁₄₇₎/ β -EKLF. These results demonstrate that a modified β -EKLF can significantly increase δ -globin gene expression.

Fig. 6 is a schematic representation of a model of δ -EKLF activation of 5 δ -globin gene expression. The δ -globin gene is normally expressed at a low level in erythroid cells. One reason for low δ -globin expression is the defective CACCC box at -90 in the δ -globin gene promoter. A modified β -EKLF, δ -EKLF, which contains zinc fingers designed to bind specifically to the defective δ -globin CACCC box, can be used in gene therapy approaches to 10 increase HbA₂ levels and inhibit erythrocyte sickling.

Detailed Description

As discussed above, the major form of adult human hemoglobin, HbA, consists of a tetramer of two α -globin chains and two β -globin chains ($\alpha_2\beta_2$). Hemoglobinopathies, such as sickle cell anemia and β^0 -thalassemia, are caused 15 by a failure of erythrocytes and erythrocyte precursors to produce normal levels of β -globin. Also as discussed above, hemoglobin A₂ (HbA₂), which consists of a tetramer of two α -globin chains and two δ -globin chains ($\alpha_2\delta_2$), is produced in low amounts in most sickle cell patients and normal adults (2-3% of total hemoglobin). HbA₂ has potent anti-sickling activity and shares some 20 functional activity with HbA. Thus, induction of increased levels of δ -globin, in order to facilitate generation of increased levels of HbA₂, can be used to treat hemoglobinopathies, such as sickle cell anemia and β^0 -thalassemia.

The CACCC box at nucleotide positions -93 to -85 in the β -globin gene promoter is important for β -globin gene expression (Dierks et al., Cell 32:695, 25 1983; Myers et al., Science 232:613, 1986; Cowie et al., Mol. Cell. Biol. 8:3122, 1988). The major protein that binds to the CACCC box of the β -globin promoter is the erythroid-specific, zinc finger transcription factor EKLF (Miller

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et al., Mol. Cell. Biol. 13:2776, 1993). β -EKLF contains three zinc fingers, which bind to the successive DNA base triplets, 5'-CCA CAC CCC-3' (nucleotides -93 to -85 with respect to the β -globin gene transcription start site), of the β -globin CACCC box. In conjunction with proteins bound to the 5 locus control region (LCR), EKLF binding to the β -globin CACCC box activates high levels of β -globin gene expression (Donze et al., J. Biol. Chem. 270:1955, 1995; Nuez et al., Nature 375:316, 1995; Perkins et al., Nature 375:318, 1995).

The human δ - and β -globin genes are located at the 3' end of the β -globin locus on chromosome 11 (Fig. 1A). Comparison of the sequences of the δ - and β -globin proximal promoters (Fig. 1B) reveals that the δ -globin promoter lacks consensus CACCC and CCAAT boxes, which are present in the β -globin promoter and are important for adult β -globin gene expression (Dierks et al., Cell 32:695, 1983; Myers et al., Science 232:613, 1986; Cowie et al., 15 Mol. Cell. Biol. 8:3122, 1988).

Sequence comparisons and analyses of crystal structures of other zinc finger-containing proteins bound to their DNA binding sites have revealed that amino acid positions in zinc finger α -helices that are involved in nucleotide-specific contacts are conserved. These conserved positions have been 20 designated -1, 2, 3, and 6, with respect to the beginning of the α -helix, and are shown for each β -EKLF zinc finger in Fig. 2. Positions 4 and 5 may also affect sequence-specific DNA recognition.

The δ -globin gene promoter contains a partial β -EKLF binding site, 5'-TGA AAC CCT-3', at nucleotide positions -89 to -81. β -EKLF does not bind to the δ -globin partial β -EKLF binding site. As is described further below, insertion of a β -EKLF binding site into the δ -globin promoter, at or near the δ -globin partial β -EKLF binding site, enhances expression from the δ -globin

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promoter. Thus, modified β -EKLFs, which bind to δ -globin promoter sequences at or near the partial β -EKLF binding site and enhance transcription from the δ -globin promoter, can be used in methods to increase δ -globin expression (Fig. 6).

5 The invention provides modified β -EKLFs, designated δ -EKLFs, that contain altered zinc finger sequences that bind to, and enhance expression from, the δ -globin promoter. δ -EKLFs of the invention bind to, for example, nucleic acids containing the partial β -globin EKLF binding site (5'-TGA AAC CCT-3') of the δ -globin promoter. Also included in the invention are δ -EKLFs
10 that bind to nucleic acids containing δ -globin nucleotides -93 to -85, which are at the same position in the δ -globin promoter, relative to the transcription start site, as the β -EKLF binding site in the β -globin promoter. Genes that encode
15 δ -EKLFs can be used in gene therapy methods for increasing HbA₂ production, which is useful in the treatment of hemoglobinopathies, such as sickle cell anemia and β^0 -thalassemia.

Production of δ -EKLFs

δ -EKLFs can be isolated, for example, using the phage display method (see, e.g., Rebar et al., Science 263:671, 1994; Smith, Science 228:1315, 1985; Wu et al., Proc. Natl. Acad. Sci. USA 92:344, 1995; Jamieson et al.,
20 Biochemistry 33:5689, 1994; Choo et al., Nature 372:642, 1994; Choo et al.,
 Proc. Natl. Acad. Sci. USA 91:11163, 1994). In this method, a hybrid gene is made in which DNA encoding a peptide or protein domain of interest, such as a peptide containing a region of random sequences, is inserted at a site in the middle portion of the gene encoding the minor coat protein, pIII, of
25 bacteriophage fd. When expressed in the bacteriophage, the portion of the resulting fusion protein containing the peptide of interest is present at the tip of the phage capsid. Phage that thus display the peptide of interest can then be

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used in selection methods for, e.g., identifying fusion proteins that contain regions that bind to specific DNA probes or antibodies. Selected phage can be amplified and sequence analysis of the selected random sequences can then be carried out. For use in isolating δ -EKLFs, the phage display method is carried 5 out as follows.

Amino acid positions -1 and 2-6 are randomized in each of the three β -EKLF zinc fingers (see Fig. 2; also see Miller et al., Mol. Cell. Biol. 13(5):2776 for the nucleotide and amino acid sequences of β -EKLF), and variants that specifically bind nucleic acids containing δ -globin promoter sequences (e.g., 5'-TGA AAC CCT-3' or 5'-CTA ATG AAA-3') are selected. Because of 10 limitations of the phage display method, preferably, one zinc finger is randomized and selected at a time. For example, beginning with the first β -EKLF zinc finger (see Fig. 2), overlapping oligonucleotides spanning the coding region of the zinc finger, and containing all four bases at the positions 15 corresponding to amino acids -1 and 2-6, are annealed and extended with DNA polymerase (e.g, Sequenase 2.0, United States Biochemical) to create a library of DNA fragments that encode a mixture of all possible amino acid combinations at amino acid positions -1 and 2-6. These fragments are linked to the second and third zinc fingers of β -EKLF, and cloned into the vector fUSES 20 (Smith et al., Meth. Enz. 217:228, 1993). The resulting random library is then transformed into an appropriate *E. coli* strain, such as MC1061 (Smith et al., Methods Enzymol. 217:228, 1993), and a collection of bacteriophage are produced, which express a modified β -EKLF in their coat protein. In these 25 modified β -EKLFs, the first zinc finger contains random sequences in amino acid positions -1 and 2-6, and the second and third zinc fingers are wild type.

Phage that display the modified β -EKLFs are then mixed with biotin-labeled double-stranded DNA fragments containing a modified β -EKLF binding site. Because the zinc fingers are being selected one at a time, the

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modified β -EKLF binding site has only one of the triplets modified. For example, in selecting the first zinc finger of δ -EKLF, the first triplet of the β -EKLF binding site is replaced with the corresponding δ -globin site. Thus, the DNA fragment contains 5'-CTA CAC CCT-3' instead of 5'-CCA CAC CCT-3', 5 or 5'-TGA CTA GGG-3' instead of 5'-ACC CTA GGG-3'. Phage containing sequences that bind to the DNA probes can be detected by contacting the phage/DNA probe mixtures with streptavidin using, e.g., a biopanning method (see, e.g., Rebar et al., *Science* 263:671, 1994).

Once the first zinc finger of a modified β -EKLF variant is selected 10 which binds to the first δ -EKLF binding site triplet, then the above-described methods are repeated to select second and third zinc fingers, which bind to the second and third δ -EKLF binding site triplets. When all three β -EKLF zinc fingers have been modified so that they each bind to their respective δ -EKLF binding site triplets, the DNA that encodes them is linked to the remainder of 15 the β -EKLF protein coding sequence, to produce a nucleic acid encoding a δ -EKLF.

In addition to phage display, other methods known in the art for 20 selecting modified zinc fingers from libraries including random sequences can be used for isolating δ -EKLFs. For example, the challenge phage method can be used. Other standard methods are described, e.g., by Ausubel et al. (*Current Protocols in Molecular Biology*, John Wiley & Sons, New York, 1994).

Expression of δ -EKLF in Hematopoietic Stem Cells

Expression of δ -EKLF in erythrocyte precursor cells (e.g., erythrocyte burst-forming cells (BFC-E) and erythrocyte colony-forming cells (CFC-E)) 25 and erythrocytes can be used to increase δ -globin production, and thus HbA₂ levels, in these cells and their progeny. In order to accomplish this, a gene encoding a δ -EKLF polypeptide can be introduced into hematopoietic stem

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cells, or erythrocyte precursor cells (e.g., BFC-E and CFC-E), using gene therapy methods known in the art. Preferably, δ -EKLF genes are stably introduced into hematopoietic stem cells, which are capable of self renewal, in order to minimize the number of treatments required. Any vectors that can be
5 used to accomplish stable integration, such as adeno-associated viral (AAV) vectors and retroviral vectors (e.g., moloney murine leukemia viral vectors), can be used. Use of an AAV vector for introduction of a gene, such as a gene encoding a δ -EKLF polypeptide, into hematopoietic stem cells can be carried out as follows. This method is also described by Luhovy et al. (Biology of
10 Blood and Marrow Transplantation 2:24-30, 1996).

AAV belongs to the genus Dependovirus (family parvoviridae), and is also known as adenovirus satellite and dependovirus. These viruses are antigenically unrelated to adenovirus, but require the presence of adenovirus in order to replicate. There are at least four serotypes of AAV, all of which share common
15 antigens. Dependoviruses are characterized in that they (1) are small and contain single-stranded DNA, (2) have virions of 18 to 26 nm in diameter, (3) are not enveloped, (4) are ether-resistant, (5) have capsids with cubic symmetry (with 32 capsomeres), and (6) replicate and assemble into virions in the nucleus of infected cells. AAV vectors for use in gene therapy methods contain AAV
20 inverted terminal repeats (ITRs).

Appropriate promoters and genes, such as genes encoding δ -EKLF polypeptides, are introduced into AAV vectors using standard methods in the art (see, e.g., Sambrook et al., eds., *Molecular Cloning: A Laboratory Manual*, 2nd Edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York,
25 1989). Any promoter that is sufficient to direct initiation of transcription in a hematopoietic cell, such as a hematopoietic stem cell or an erythrocyte precursor cell, can be used in the invention. For example, a preferred non-tissue specific promoter is the cytomegalovirus (CMV) promoter (DeBernardi

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et al., Proc. Natl. Acad. Sci. USA 88:9257-9261, 1991, and references therein). Any appropriate hematopoietic cell-type specific promoter may also be used in the invention. For example, globin promoters, such as the β -globin promoter (Karlsson et al., Ann. Rev. Biochem. 54:1071-1108, 1985, and references therein) (e.g., a β -globin promoter including a DNase hypersensitivity site, such as HS2), may be used.

The hematopoietic stem cells can be obtained from any appropriate source. Preferably, the cells are obtained from the patient into which they are to be transplanted after AAV-infection. Hematopoietic stem cells are obtained from patients and, after infection by AAV vectors, are introduced back into patients using standard methods in the art (see, e.g., United States Patent 5,061,620; Rubenstein et al., (eds.) Scientific American Medicine, Scientific American, Inc. (New York, 1978), and references therein).

Production of AAV vectors containing genes encoding δ -EKLF polypeptides can be carried out using the semi-packaging cell line and vectors described below.

Production of high titer, recombinant AAV

A packaging cell line can be used to produce a high titer (10^7 - 10^8 pfu/ml) stock of AAV-derived vectors containing genes encoding a δ -EKLF polypeptide (e.g., a δ -EKLF gene functionally linked to a CMV promoter). Such a "semi-packaging" cell line can be established with a silent episomal vector, such as pEBAVrc. This vector contains an EBV replication origin and an EBNA1 gene. This arrangement enables the vector to be established as an episome and to replicate to high copy numbers. The rep-cap genes of AAV are inserted into the vector with their natural control elements, so these genes are activated only in the presence of adenovirus early gene products. This arrangement provides a switch-system to regulate the expression of the rep

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gene, which has been found to have a cytostatic effect. The vector also contains a hygromycin resistance gene to enable selection for cells that contain the vector. The plasmid sequences of the vector contain an ampicillin resistance gene and a bacterial replication origin for replicating the DNA in
5 bacteria.

An AAV vector containing a δ-EKLF gene can be made in a similar vector, to generate pEBAVcmv/δ-EKLF. This vector contains a CMV immediate-early transcriptional cassette containing a δ-EKLF gene, which is flanked by the two ITR sequences of AAV.

10 To establish the semi-packaging cell line, HeLa cells in 100 mm culture dishes are transfected with pEBAVrc. HeLa cells are seeded 24 hours prior to transfection, and are allowed to reach 50-70% confluence at the time of transfection. Cells are washed once with serum-free DMEM (Gibco, Gaithersburg, MD). After removal of the medium, 8 ml of DMEM containing
15 10% fetal calf serum is added to the culture on the next day. At 12 hours after this medium change, cells are treated with trypsin and passed to three 100 mm dishes in DMEM containing 10% fetal calf serum and 270 u/ml of Hygromycin (Sigma). Special care should be taken to allow cells to settle at the bottom of the plate individually. Cells are selected with hygromycin for 10 to 15 days, or
20 until individual colonies begin to grow. Colonies from 3 to 4 dishes are treated with trypsin and pooled into one 100 mm dish. Cells are kept at 60-70% confluence in medium containing 200 u/ml hygromycin. When 90% confluence is reached, cells are transferred into four 100 mm dishes in DMEM containing 10% DMEM without hygromycin. At 24 hours post-seeding, each
25 100 mm dish of cells is transfected with 5 µg each of pEBAVrc and pEBAVcmv/δ-EKLF. At 3 hours after the addition of the transfection medium, wild-type adenovirus (HuAd5) is added to the cells at an MOI of 10, without removal of the transfection medium. At 12 hours after the transfection, the

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medium is changed to DMEM containing 5% fetal calf serum and incubated for three days, or until most of the cells show cytopathic effects. All of the media, except 1 ml, is removed from each 100 mm dish. Cells are then scraped off the plate and transferred into a 10 ml screw-cap tube. This viral lysate is stored at -5 100°C, until further treatment.

The viral lysate is thawed on ice, and sonicated for 10-15 minutes with pulse ultrasound. The tube is put back on ice periodically to prevent the sample from heating up. The viral lysate is then spun at 15,000 rpm for 10 minutes in a JL-20 centrifuge (Beckman). The supernatant is removed and aliquoted into 10 freezing wells of 10 ml each and stored at -100°C. For infecting hematopoietic stem cells, it is not necessary to remove or inactivate the adenovirus, since stem cells lack receptors for adenovirus. For infection of cells that are susceptible to adenovirus infection, adenovirus should be removed by chromatography or by CsCl gradient centrifugation.

15 The titer of the virus can be determined by the rate of transduction of HeLa cells with the δ-EKLF gene. One, two, and three ml of the viral stock can be used to infect HeLa cells in 24-well plates. At 24 hours post-infection, the cells can be fixed with 0.1% glutaraldehyde at 4°C for 2 minutes, and washed thoroughly with DMEM. Cells can then be monitored for δ-EKLF expression, using standard methods. The transfection procedure is as follows. 20 For each 100 mm dish, 8-10 µg DNA is dissolved in 2 ml of DMEM or transfection medium from BRL. 90-100 µl of lipofectamine (BRL) is suspended in 2 ml of DMEM. The lipid containing medium is mixed with the DNA containing medium slowly and dropwise, while the tube is gently rotated. 25 After mixing well by gently rotating the tube, the transfection mixture is incubated for 30 minutes at room temperature. The mixture is then diluted with DMEM to a total volume of 8 ml. The mixture is then added to the cells, which

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had been washed once or twice with DMEM without serum. Care should be taken to not allow the cells to dry between each wash.

Purification of human hematopoietic stem cells

Bone marrow is obtained from individuals following an IRB approved protocol. Mononuclear cells are purified using density gradient centrifugation using standard procedures. The mononuclear bone marrow cells are isolated by Ficoll/Hypaque density gradient centrifugation (average yield $1-2 \times 10^8$ cells). The use of magnetic activated cell sorting (MACS) allows for rapid enrichment of CD34+ cells (Kato and Radbruch, Cytometry 14:384-392, 1993). The cells are labeled with anti-CD34+ antibodies conjugated with magnetic particles, and run through the magnetic separation column from Miltenyi Biotec (Sunnyvale, CA). Hematopoietic stem cells can be further enriched by selecting for and against various surface molecules according to principles outlined by Baum et al. (Proc. Natl. Acad. Sci. USA 89:2804-2808, 1992). The progenitors can be labeled with an FITC conjugated antibody that recognizes a different CD34+ epitope than the magnetic bead conjugated anti-CD34 antibody. Lin- cells are isolated by a negative selection using a cocktail of PE labeled antibodies that recognize myeloid, B cells, T cells, and NK cell markers (CD 2, CD 14, CD 19, CD 56). Thy+ cells are isolated by positive selection with biotinylated, anti-human Thy mouse monoclonal antibody that was labeled with streptavidin-conjugated, Texas red dye.

Infection of hematopoietic stem cells

Ten thousand CD34+, Lin-, and Thy+ cells are suspended in 200 μ l of DMEM without serum and are dispensed into a single well of a 96 well plate. Ten microliters of the recombinant virus (AAV cmv/ δ -EKLF; 10^8 u/ml) are then added to the cells and incubated at 37°C for 16 hours. The cells are then

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washed 3 times with DMEM without serum and can be plated into long term culture as described by Eaves et al. (J. Tiss. Cult. Meth. 13:55-62, 1991) for further analysis.

Analysis of cells plated into long term culture

5 After 6 weeks in long term culture, cells are removed and plated in methylcellulose for clonogenic progenitor assays (Eaves and Eaves, Blood 52:1196-1210, 1978). BFU-E are picked after 14 days, DNA is extracted, and
PCR is performed with primers specific for δ-EKLF. PCR products are run on agarose gels, blotted to nitrocellulose, and hybridized with radiolabeled, δ-
10 EKLF sequences.

δ-EKLF Polypeptide Expression

δ-EKLF polypeptides can be used to produce antibodies against δ-EKLF. Such antibodies can be used to monitor the levels of expression of δ-EKLF in patients treated by the gene therapy methods of the invention. In
15 general, δ-EKLF polypeptides can be produced by transformation of a suitable host cell with all or part of a δ-EKLF-encoding nucleic acid fragment (e.g., a cDNA fragment) in a suitable expression vehicle.

Those skilled in the field of molecular biology will understand that any of a wide variety of expression systems can be used to provide the recombinant protein. The precise host cell used is not critical to the invention. The δ-EKLF protein can be produced in a prokaryotic host (e.g., *E. coli*) or in a eukaryotic host (e.g., *Saccharomyces cerevisiae*, insect cells, e.g., Sf21 cells, or mammalian cells, e.g., COS-1, NIH 3T3, or HeLa cells). Such cells are available from a wide range of sources (e.g., the American Type Culture
20 Collection, Rockland, MD; also, see, e.g., Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, 1994). The method of
25

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transformation or transfection and the choice of expression vehicle will depend on the host system selected. Transformation and transfection methods are described, e.g., in Ausubel et al. (*supra*); expression vehicles can be chosen from those provided, e.g., in *Cloning Vectors: A Laboratory Manual* (P.H.

5 Pouwels et al., 1985, Supp. 1987).

One particular expression system is the baculovirus system (using, for example, the vector pBacPAK9) available from Clontech (Pal Alto, CA). Alternatively, a δ-EKLF polypeptide can be produced by a stably-transfected mammalian cell line. A number of vectors suitable for stable transfection of
10 mammalian cells are available to the public, e.g., see Pouwels et al. (*supra*); methods for constructing such cell lines are also publicly available, e.g., in Ausubel et al. (*supra*). In one example, cDNA encoding the δ-EKLF polypeptide is cloned into an expression vector which includes the dihydrofolate reductase (DHFR) gene. Integration of the plasmid and,
15 therefore, the δ-EKLF polypeptide-encoding gene into the host cell chromosome is selected for by including 0.01-300 μM methotrexate in the cell culture medium (as described in Ausubel et al., *supra*). This type of dominant selection can be accomplished in most cell types. Recombinant protein expression can be increased by DHFR-mediated amplification of the
20 transfected gene. Methods for selecting cell lines bearing gene amplifications are described in Ausubel et al. (*supra*); such methods generally involve extended culture in medium containing gradually increasing levels of methotrexate. DHFR-containing expression vectors commonly used for this purpose include pCVSEII-DHFR and pAdD26SV(A) (described in Ausubel et
25 al., *supra*). Any of the host cells described above or, preferably, a DHFR-deficient CHO cell line (e.g., CHO DHFR⁻ cells, ATCC Accession No. CRL 9096) are among the host cells preferred for DHFR selection of a stably-transfected cell line or DHFR-mediated gene amplification.

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Once the recombinant δ -EKLF polypeptide is expressed, it can be isolated, e.g., using affinity chromatography. In one example, an anti- δ -EKLF polypeptide antibody (e.g., produced as described herein) can be attached to a column and used to isolate the δ -EKLF polypeptide. Lysis and fractionation of 5 δ -EKLF polypeptide-harboring cells prior to affinity chromatography can be performed by standard methods (see, e.g., Ausubel et al., *supra*). Once isolated, the recombinant protein can, if desired, be further purified, e.g., by high performance liquid chromatography (see, e.g., Fisher, *Laboratory Techniques In Biochemistry And Molecular Biology*, eds., Work and Burdon, 10 Elsevier, 1980).

Polypeptides of the invention, particularly short δ -EKLF polypeptide fragments (e.g., fragments containing zinc fingers), can also be produced by chemical synthesis (e.g., by the methods described in *Solid Phase Peptide Synthesis*, 2nd ed., 1984, The Pierce Chemical Co., Rockford, IL).

15 These general techniques of polypeptide expression and purification can also be used to produce and isolate useful δ -EKLF fragments (e.g., fragments containing one or more zinc fingers, or portions thereof) or analogs.

Antibodies against δ -EKLF

To generate δ -EKLF-specific antibodies, a δ -EKLF coding sequence 20 (e.g., a sequence encoding a δ -EKLF zinc finger DNA binding domain) can be expressed as a C-terminal fusion with glutathione S-transferase (GST) (Smith et al., Gene 67:31-40, 1988). The fusion protein can be purified on glutathione-Sepharose beads, eluted with glutathione cleaved with thrombin (at a engineered cleavage site), and purified to the degree necessary for 25 immunization of rabbits.

Primary immunizations can be carried out with Freund's complete adjuvant and subsequent immunizations with Freund's incomplete adjuvant.

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Antibody titers can be monitored by Western blot analysis and immunoprecipitation using the thrombin-cleaved δ -EKLF protein fragment of the GST- δ -EKLF fusion protein. Immune sera can be affinity purified using a CNBr-Sepharose-coupled δ -EKLF polypeptide. Antiserum specificity is
5 determined using a panel of unrelated GST proteins.

As an alternate or adjunct immunogen to GST fusion proteins, peptides corresponding to relatively unique hydrophilic regions of δ -EKLF can be generated and coupled to keyhole limpet hemocyanin (KLH) through an introduced C-terminal lysine. Antiserum to each of these peptides can be
10 similarly affinity purified on peptides conjugated to BSA, and specificity tested in ELISA and Western blots using peptide conjugates, and by Western blot and immunoprecipitation using δ -EKLF expressed as a GST fusion protein.

Alternatively, monoclonal antibodies can be prepared using δ -EKLF polypeptides and standard hybridoma technology (see, e.g., Kohler et al.,
15 Nature 256:495, 1975; Kohler et al., Eur. J. Immunol. 6:511, 1976; Kohler et al., Eur. J. Immunol. 6:292, 1976; Hammerling et al., *In Monoclonal Antibodies and T Cell Hybridomas*, Elsevier, NY, 1981; Ausubel et al., *supra*). Once produced, monoclonal antibodies can also be tested for specific δ -EKLF recognition by Western blot or immunoprecipitation analysis (e.g., by the
20 methods described in Ausubel et al., *supra*). Antibodies that specifically recognize δ -EKLF are considered to be useful in the invention; such antibodies can be used, e.g., in an immunoassay to monitor the level of δ -EKLF produced by a mammal (e.g., a human treated using a gene therapy method of the invention).

25 Preferably, antibodies of the invention are produced using fragments of the δ -EKLF polypeptide that appear likely to be antigenic, as determined by criteria such as high frequency of charged residues. In one specific example, such fragments are generated by standard techniques of PCR and cloned into

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the pGEX expression vector (Ausubel et al., *supra*). Fusion proteins are expressed in *E. coli* and purified using a glutathione agarose affinity matrix as described in Ausubel et al. (*supra*). To attempt to minimize the potential problems of low affinity or specificity of antisera, two or three such fusions are 5 generated for each protein, and each fusion is injected into at least two rabbits. Antisera are raised by injections in a series, preferably, including at least three booster injections.

Insertion of a β-EKLF Binding Site Activates Transcription of the δ-Globin Promoter

10 Experiments demonstrating that the presence of the β-EKLF binding site leads to activation of a downstream δ-globin promoter were carried out as follows.

Plasmid constructions

The construction of plasmid HS2 β/Luciferase has been described 15 (Donze et al., J. Biol. Chem. 270:1955, 1995). The plasmid contains the 1.5 kb *Kpn*I-*Bgl*II human Locus Control Region HS2 fragment upstream of the -265 to +48 human β-promoter driving the Luciferase reporter gene of plasmid pGL2-Basic (Promega Corp.). HS2 δ/Luciferase was constructed by PCR amplification of the -265 to +48 region of the δ-promoter with *Bgl*II ends attached to the primers. The template used was a human globin locus subclone containing the entire δ-gene, and the primer sequences (*Bgl*II sites underlined) 20 were: upstream 5'-CTCGAGGCTAGCAGATTGTCAAAATGAAACTAGA-3' (SEQ ID NO:1); downstream, 5'-
CTCGAGGCTAGCAGATTCTGTTGAGGTTGCTAGTGA-3' (SEQ ID
25 NO:2). The PCR product was digested with *Bgl*II and cloned into the plasmid HS2/Luc (Caterina et al., Nucleic Acids Res. 22:2383, 1994). Modified δ^{CAC-1}

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and $\delta^{\text{CAC-2}}$ promoters were made by the megaprimer mutagenesis method (Sarkar et al., BioTechniques 8:404, 1990; Barik et al., BioTechniques 10:489, 1991; Aiyar et al., BioTechniques 14:366, 1993), using the same outside oligonucleotides listed above with the following mutagenic oligonucleotides:

5 $\delta^{\text{CAC-1}}$, 5'-

TTTCATTCTCACAAACTA**ACCACACCCT**GCTTATCTTAAAC

CA-3' (SEQ ID NO:3); $\delta^{\text{CAC-2}}$, 5'-

TCATTTTCATTCTCACAAACCACACCC

TCCCTGCTTATCTTAAACCAA-3' (SEQ ID NO:4). The consensus EKLF

10 binding sites are underlined and in bold, and these fragments were cloned into HS2/Luc, as is described above.

To construct the HS2 δ - β gene plasmids, the 3.0 kb *Xba*I-*Sph*I fragment of the δ -globin gene (from -400 to about 1 kb downstream of the poly A site) was first subcloned into pUC19. The modified δ -promoters (amplified from the -400 *Xba*I site to the *Sall* site at -13) were then synthesized by megaprimer mutagenesis using the same mutagenic oligos listed above and outside primers 5'-AGTTAAACTGCAGCAATAG-3' (SEQ ID NO:5) (starting at -460 of the δ -promoter), and 5'-CTTCTCCTCAGGAGTCAG-3' (SEQ ID NO:6), (just downstream of the translation start site). These PCR fragments were cut with *Xba*I and *Sall*, and used to replace the corresponding wild type fragment in the δ -gene pUC19 subclone. The δ^{GAL4} -promoter was constructed in the same fashion, using the mutagenic oligonucleotide 5'-

GAAGGTTCATTTTCATTCT**CCGGAGGA**

CAGTCCTCCGGCTTATCTTAAACCAACCTGC-3' (SEQ ID NO:7), with

25 the consensus GAL4 binding site (Giniger et al., Cell 40:767, 1985) underlined and in bold. Each of these δ -genes was cut out of the pUC19 vector with *Xba*I and *Hind*III, blunted with S1 nuclease, and cloned into plasmid 5'HS2 (K-P) β^{22} (Caterina et al., Nucleic Acid Res. 22:1006, 1994), which was cut with *Clal*

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and blunted with S1 nuclease. All PCR amplified regions in each of these constructs were completely verified by dideoxy sequencing using the Sequenase kit (United States Biochemical).

The pCIneo-GAL4₍₁₋₁₄₇₎/EKLF plasmid was constructed in two steps: A 5 0.8 kb *NcoI-MsI* cDNA fragment containing the coding sequence (amino acids 2-275) of the murine EKLF activation domain (from plasmid pSG5-EKLF (Miller et al., Mol. Cell. Biol. 13:2776, 1993)) was blunted with S1 nuclease and cloned in frame into the GAL4 DNA binding domain expression plasmid pBXG1. The pBXG1/EKLF plasmid was cut with *HindIII* and *BamHI*, the 1.3 10 kb GAL4₍₁₋₁₄₇₎/EKLF fragment was isolated, blunted with S1 nuclease, and cloned into pCIneo (Promega Corp.) cut with *SmaI*. As a control, the 0.5 kb GAL4 binding domain *HindIII-BamHI* fragment from pBXG1 was cloned into pCIneo, as is described above (pCIneo-GAL4₍₁₋₁₄₇₎).

Mouse erythroleukemia (MEL) cell transfections

15 MEL cells were maintained in DMEM containing 10% fetal bovine serum. For transient transfections, cells were washed once and resuspended to 10⁸ cells/ml in RPMI 1640 medium without serum. For transient luciferase assays, 40 µg of HS2 promoter/Luciferase plasmids were mixed with 5 µg internal control plasmid CMV-βgal (Clontech) in 100 µl phosphate buffered 20 saline (PBS) and placed in a 0.4 cm gap electroporation cuvette (Bio-Rad). 5x10⁷ MEL cells in 0.5 ml RPMI 1640 medium (without serum) were added to each cuvette, and the cells were electroporated at 260 V and 960 µF in a Bio-Rad Gene Pulser. The contents of each cuvette were transferred into a 100 mm plate containing 10 ml DMEM/10% FBS, and incubated for 8 hours. After 8 25 hours, DMSO was added to 1.65% to induce erythroid differentiation. This 8 hour pre-incubation, before DMSO induction, increased cell viability and gene expression levels several fold when compared to cells induced immediately

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after electroporation. Cultures were incubated for three days and then cell extracts were assayed for luciferase and β -galactosidase as described (Caterina et al., Nucleic Acids Res. 22:2383, 1994; Donze et al., J. Biol. Chem. 270:1955, 1995). Transient transfections were performed three times in 5 triplicate.

Transfection of MEL cells to produce G418 resistant populations was performed essentially as described by Collis et al. (EMBO J. 9:233, 1990). Twenty micrograms of HS2 δ - β plasmids (with δ , δ^{CAC-1} , or δ^{CAC-2} promoters) were mixed with a 1/10 molar ratio of plasmid pgk-neo (the murine 10 phosphoglycerate kinase promoter driving neomycin phosphotransferase). Both plasmids were linearized with *Kpn*I, precipitated, and dissolved in 100 μ l PBS. The plasmid mix was placed into a 0.4 cm electroporation cuvette, and 2x10⁷ MEL cells (prepared as is described above) in 0.5 ml were added. Cells were electroporated at 200 V and 960 μ F, and transferred to 30 ml DMEM/10% 15 FBS in a 75 cm² culture flask. After 48 hours, G418 was added to a final concentration of 300 μ g/ml, and resistant cell populations were selected for two weeks. Cells were then induced with 1.65% DMSO for three days, and total RNA was extracted as described (Chomczynski et al., Anal. Biochem. 162:156, 1987). The HS2 δ^{GAL4} - β experiments were performed as above, using a 1:10 20 molar ratio of the pClineo plasmids, which were linearized with *Xmn*I.

Analysis of relative levels of δ - and β -transcripts

The relative levels of δ - to β -globin transcripts were determined by the Single Nucleotide Primer Extension (SNuPE) assay, which is based on detecting single known nucleotide differences in allelic RNAs (Szabo et al., 25 Genes Dev. 9:1857, 1995). Briefly, RNA from MEL cells transfected with HS2 δ - β constructs was amplified by RT-PCR (cDNA cycle kit, Invitrogen)

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using oligo dT to prime the cDNA reaction. The PCR primers were: upstream, 5'-TGTTCACTAGCAA CCTCAAAC-3' (SEQ ID NO:8); and downstream, 5'-TGAAGTTCTCAGGAT CCACGT-3' (SEQ ID NO:9). There are no differences in the δ- and β-globin sequences in these regions and the primers do not amplify mouse globin mRNAs. These oligos specifically amplify corresponding 341 bp fragments of both the human δ- and β-globin cDNAs, and the fragments were purified by agarose gel electrophoresis. The detection oligo, 5'- CTTCTCCTCAGGAGTCAG-3' (SEQ ID NO:6), hybridizes to identical sense strand sequences of both δ- and β-globin cDNA PCR products (corresponding to nucleotides +77 to +60 of each message), and is extended to base +59 by α³²P-dATP in the δ reaction or by α³²P-dGTP for the β reaction. Labeled products were resolved on a 15% polyacrylamide/8M urea gel, and bands were quantitated on a Molecular Dynamics Model 425 Phosphorimager. Linearity of the assay was verified as described (Szabo et al., Genes Dev. 9:1857, 1995) by mixing experiments using RNA from MEL cells transfected with either HS2 β or HS2 β-promoter/δ-gene as sources containing the individual messages.

Experimental Results

To test the feasibility of using a modified EKLF to activate δ-globin gene expression, we determined whether a δ-globin promoter containing a β-globin EKLF binding site would increase expression by recruiting endogenous cellular β-EKLF in transient transfection assays. We replaced the defective β-EKLF binding site in the δ-globin promoter (TGAAACCCT from -89 to -81, Fig. 1B) with the consensus CCACACCCT, to construct the plasmid HS2 δ^{CAC-1}/Luciferase. We also constructed a modified δ-globin promoter that contained the CCACACCCT sequence at -93 to -85, so that the β-EKLF

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binding site would be positioned the same distance from the transcription start site as it is in the β -globin promoter (HS2 δ^{CAC-2} /Luciferase).

Fig. 3B illustrates the relative expression levels of these modified δ -promoter/Luciferase reporter constructs (Fig. 3A) when linked to a Locus Control Region HS2 fragment and transiently transfected into mouse erythroleukemia (MEL) cells. In this assay, HS2 δ /Luc was expressed at 10% of the level of HS2 β /Luc. When the consensus β -EKLF site was placed in the δ -globin promoter at the -81 to -89 site (HS2 δ^{CAC-1} /Luc), expression was increased approximately 5 fold, to 56% of HS2 β /Luc expression. HS2 δ^{CAC-2} /Luc, with the consensus β -EKLF site at -85 to -93, expressed the reporter gene at 99% of HS2 β /Luc. These results demonstrate that the major defect of the δ -globin gene proximal promoter is the lack of a consensus β -EKLF binding site.

In addition to promoter defects, low δ -globin expression has been attributed to other differences between the δ - and β -globin genes. The δ -gene lacks intragenic enhancers, which augment β -globin expression (Kosche et al., Nucleic Acids Res. 13:7781, 1985; Behringer et al., Proc. Natl. Acad. Sci. USA 84:7056, 1987), and δ -globin mRNA is less stable than β -globin mRNA (Ross et al., J. Mol. Biol. 167:607, 1983). To assess the effects of these differences on δ^{CAC-1} and δ^{CAC-2} expression, we constructed complete δ -globin genes (see methods) containing the δ , δ^{CAC-1} , and δ^{CAC-2} promoters (Fig. 4A). These δ -globin genes were used to produce HS2 δ - β constructs. The plasmids were then co-transfected into MEL cells with a pgk-neo selectable marker and G418 resistant populations were selected. After differentiation was induced with DMSO, RNA was extracted and the relative levels of δ - and β -globin mRNAs were determined by the Single Nucleotide Primer Extension assay (SNuPE) (Szabo et al., Genes Dev. 9:1857, 1995). Table I shows the results of these experiments. The level of δ -globin mRNA ($\delta/\delta+\beta$) in MEL cells was 3.0 ±

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1.3% for HS2 δ - β , $14.9 \pm 7.4\%$ for HS2 $\delta^{\text{CAC-1}}$ - β , and $22.0 \pm 9.0\%$ for HS2 $\delta^{\text{CAC-2}}$ - β . These results demonstrate that insertion of a consensus β -EKLF binding site in the δ -globin promoter significantly enhances δ -globin gene expression in a construct that contains the entire δ -globin gene. A 5 representative example of the SNuPE assay is illustrated in Fig. 4B; $\delta/\delta+\beta$ -globin mRNA in this experiment was 3.6% for HS2 δ - β , 24.7% for HS2 $\delta^{\text{CAC-1}}$ - β , and 34.8% for HS2 $\delta^{\text{CAC-2}}$ - β . As a control, δ - and β -globin mRNA levels were determined for human reticulocyte RNA (Fig. 4B); the $\delta/\delta+\beta$ level was $0.6 \pm 0.2\%$.

10 These results show that β -EKLF binding to the δ -globin gene promoter significantly stimulates expression; however, the experiments do not exclude the possibility that other CACCC binding factors are responsible for this increase. To confirm that the β -EKLF activation domain can enhance δ -globin gene expression, we constructed an HS2 δ^{GAL4} - β plasmid in which the 15 consensus GAL4 binding site replaced sequences from -80 to -99 of the δ -globin gene promoter (Fig. 5A). The coding sequence of the murine β -EKLF activation domain (amino acids 2-275) was fused in frame to the GAL4 DNA binding domain (amino acids 1-147), and the fusion fragment was subcloned into the pCIneo expression vector (Fig. 5B). MEL cells were co-transfected 20 with HS2 δ^{GAL4} - β plus pCIneo-GAL4₍₁₋₁₄₇₎ or HS2 δ^{GAL4} - β plus pCIneo-GAL4₍₁₋₁₄₇₎/ β -EKLF. Stable pools of G418 resistant cells were selected and, after DMSO induction, δ - and β -globin mRNA levels were determined by the SNuPE assay described above.

The data in Table 2 demonstrate that the level of human $\delta/\delta+\beta$ mRNA is 25 $9.9 \pm 2.5\%$ in cells containing HS2 δ^{GAL4} - β plus pCIneo-GAL4₍₁₋₁₄₇₎ and $27.8 \pm 7.1\%$ in cells containing HS2 δ^{GAL4} - β plus pCIneo-GAL4₍₁₋₁₄₇₎/ β -EKLF. A representative example is illustrated in Fig. 5C; the level of human $\delta/\delta+\beta$ mRNA is 4.4% in cells containing HS2 δ - β plus pCIneo-GAL4₍₁₋₁₄₇₎, 13.3% in

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cells containing HS2 δ^{GAL4} - β plus pCIneo-GAL4₍₁₋₁₄₇₎, and 36.6% in cells containing HS2 δ^{GAL4} - β plus pCIneo-GAL4₍₁₋₁₄₇₎/ β -EKLF. These results demonstrate that a modified β -EKLF can significantly increase δ -globin gene expression.

5 The 3-fold increase (4.4% to 13.3%) of δ -globin gene expression observed when δ promoter sequences from -80 to -99 are replaced with a GAL4 binding site suggests that this region binds a factor that represses expression. A 3-fold increase is also observed when the -80 to -99 region of the δ -globin promoter is deleted in an HS2 δ - β globin construct.

10 **Table 1. Relative Levels of δ -globin Transcripts From MEL Transfections and Human Reticulocyte Samples as % of Total Human $\delta + \beta$ Transcripts.**

| | | <u>HS2 δ-β</u> | <u>HS2 δ^{CAC-1}-β</u> | <u>HS2 δ^{CAC-2}-β</u> | <u>Reticulocyte</u> |
|----|--------|---|---|---|---------------------|
| | Exp. 1 | 3.6 | 24.7 | 34.8 | 0.5 |
| 15 | Exp. 2 | 1.9 | 10.0 | 15.0 | 0.7 |
| | Exp. 3 | 1.6 | 5.8 | 12.3 | 0.8 |
| | Exp. 4 | 4.7 | 19.0 | 25.7 | 0.3 |
| | Avg. | 3.0 +/- 1.3 | 14.9 +/- 7.4 | 22.0 +/- 9.0 | 0.6 +/- 0.2 |

20

Expression of modified δ -globin genes in MEL cells stably transfected with HS2 δ - β constructs. These constructs were transfected into MEL cells with a pgk-neo selectable marker, and G418 resistant populations were selected. The cells were induced to differentiate with DMSO, and RNA was extracted for analysis. the relative levels of δ - and β -globin mRNAs were determined by the Single Nucleotide Primer Extension (SNuPE) assay and quantitated by phosphorimager analysis.

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Table 2. Relative Levels of δ -globin Transcripts From MEL Cells Transfected with HS2- δ^{GAL4} - β and GAL4₍₁₋₁₄₇₎ Expression Constructs as % of Total $\delta + \beta$ Transcripts

| | | HS2 δ^{GAL4} - β <u>+GAL4₍₁₋₁₄₇₎</u> | HS2 δ^{GAL4} β <u>+GAL4₍₁₋₁₄₇₎/β-EKLF</u> |
|----|--------|---|--|
| 5 | Exp. 1 | 13.3 | 36.6 |
| | Exp. 2 | 8.4 | 30.2 |
| | Exp. 3 | 6.7 | 16.8 |
| 10 | Exp. 4 | 11.3 | 27.3 |
| | Avg. | 9.9 +/- 2.5 | 27.8 +/- 7.1 |

GAL4/β-EKLF activates δ -globin gene expression in an HS2 δ GAL4- β construct. HS2 δ GAL- β was stably transfected into MEL cells with either pCIneo-GAL4₍₁₋₁₄₇₎ or pCIneo-GAL4₍₁₋₁₄₇₎/β-EKLF, and relative transcript levels were determined by the SNuPE assay. These results demonstrate that a modified β-EKLF can significantly increase δ -globin gene expression.

All publications referred to herein are incorporated by reference in their entirety. Other embodiments are in the following claims.

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SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT: The UAB Research Foundation

(ii) TITLE OF THE INVENTION: DELTA-ERYTHROID KRÜPPEL-LIKE
FACTORS AND METHODS OF USE

(iii) NUMBER OF SEQUENCES: 17

(iv) CORRESPONDENCE ADDRESS:

- (A) ADDRESSEE: Clark & Elbing LLP
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- (C) CITY: Boston
- (D) STATE: MA
- (E) COUNTRY: USA
- (F) ZIP: 02110

(v) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Diskette
- (B) COMPUTER: IBM Compatible
- (C) OPERATING SYSTEM: DOS
- (D) SOFTWARE: FastSEQ for Windows Version 2.0

(vi) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER: PCT/US97/----
- (B) FILING DATE: 13-JUN-97
- (C) CLASSIFICATION:

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- (A) APPLICATION NUMBER: 60/019,769
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 35 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

-31-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTCGAGGCTA GCAGATCTGC AAAATGAAA CTAGA

35

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CTCGAGGCTA GCAGATCTCT GTTGAGGTT GCTAGTGA

38

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 44 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TTTCATTCT CACAAACTAA CCACACCCCTG CTTATCTTAA ACCA

44

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 49 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TCATTTTCA TTCTCACAAA CCACACCCCTC CCTGCTTATC TTAAACCAA

49

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

-32-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AGTTTAAACT GCAGCAATAG

20

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CTTCTCCTCA GGAGTCAG

18

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 59 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GAAGGGTTCAT TTTTCATTCT CCGGAGGACA GTCCTCCGGC TTATCTTAAA CCAACCTGC

59

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TGTTCACTAG CAACCTCAAA C

21

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

-33-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TGAAGTTCTC AGGATCCACG T

21

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 37 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TGTGGAGCCA CACCCTAGGG TTGGCCAATC TACTCCC

37

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 37 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TCACAAACTA ATGAAACCCCT GCTTATCTTA AACCAAC

37

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 37 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TCACAAACTA ACCACACCCCT GCTTATCTTA AACCAAC

37

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 37 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

-34-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TCACAAACCA CACCCTCCCT GCTTATCTTA AACCAAC

37

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Cys Gly His Glu Gly Cys Gly Lys Ser Tyr Ser Lys Ser Ser His Leu
1 5 10 15
Lys Ala His Leu Arg Thr His
20

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Cys Ser Trp Asp Gly Cys Asp Trp Arg Phe Ala Arg Ser Asp Glu Leu
1 5 10 15
Thr Arg His Tyr Arg Lys His
20

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Cys Gly Leu Cys Gly Leu Cys Pro Arg Ala Phe Ser Arg Ser Asp His
1 5 10 15
Leu Ala Leu His Met Lys Arg His
20

-35-

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

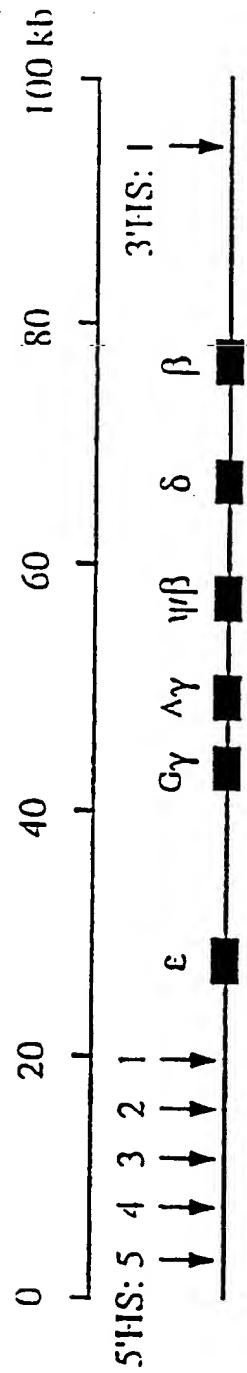
CCGGAGGACA GTCCTCCGG

19

-36-

1. Use of a nucleic acid encoding a δ-EKLF polypeptide in the preparation of a medicament for inducing δ-globin gene expression in a cell.
2. A δ-EKLF polypeptide.
3. A nucleic acid encoding a δ-EKLF polypeptide.
- 5 4. A cell that contains a nucleic acid encoding a δ-EKLF polypeptide.
5. The cell of claim 4, wherein said cell is a hematopoietic stem cell.
6. The cell of claim 5, wherein said cell is an erythrocyte precursor cell.
7. A vector containing a nucleic acid encoding a δ-EKLF polypeptide.
8. The vector of claim 7, wherein said vector is an adeno-associated viral
10 (AAV) vector or a retroviral vector.
9. A method for identifying a δ-EKLF polypeptide, said method comprising contacting a nucleic acid containing the sequence 5'-TGA AAC CCT-3' or the sequence 5'-CTA ATG AAA-3' with a candidate polypeptide that has been modified such that it differs from wild type β-EKLF by at least one amino acid and
15 identifying said polypeptide by its ability to bind to said nucleic acid.
10. The method of claim 9, wherein said amino acid is in a β-EKLF zinc finger that binds DNA.

FIG. 1A



(seq ID no: 10) β TGTGGAGCCACACCCTAGGGTTGCCAAATCTACTCCC
-93 -85

(seq ID no: 11) δ TCACAAACTAATGAAACCCCTGCTTATCTTAAACCAAC
-85 -81

(seq ID no: 12) δ_{CAC} TCACAAACTAACCCACACCCCTGCTTATCTTAAACCAAC
-89 -81

(seq ID no: 13) δ_{CAC} TCACAAACCCACACCCCTGCTTATCTTAAACCAAC
-93 -85

FIG. 1B

FIG. 2

FINGER 1 CGHEGGCGKSYSKSSHLKAHLRTH -1 23456 (SEQ ID NO: 14)

FINGER 2 CSWDGCDWRFARSDDELTRHYRKH -1 23456 (SEQ ID NO: 15)

FINGER 3 CGLCGLCPRAFSRSDHLALHMKRH -1 23456 (SEQ ID NO: 16)

FIG. 3A

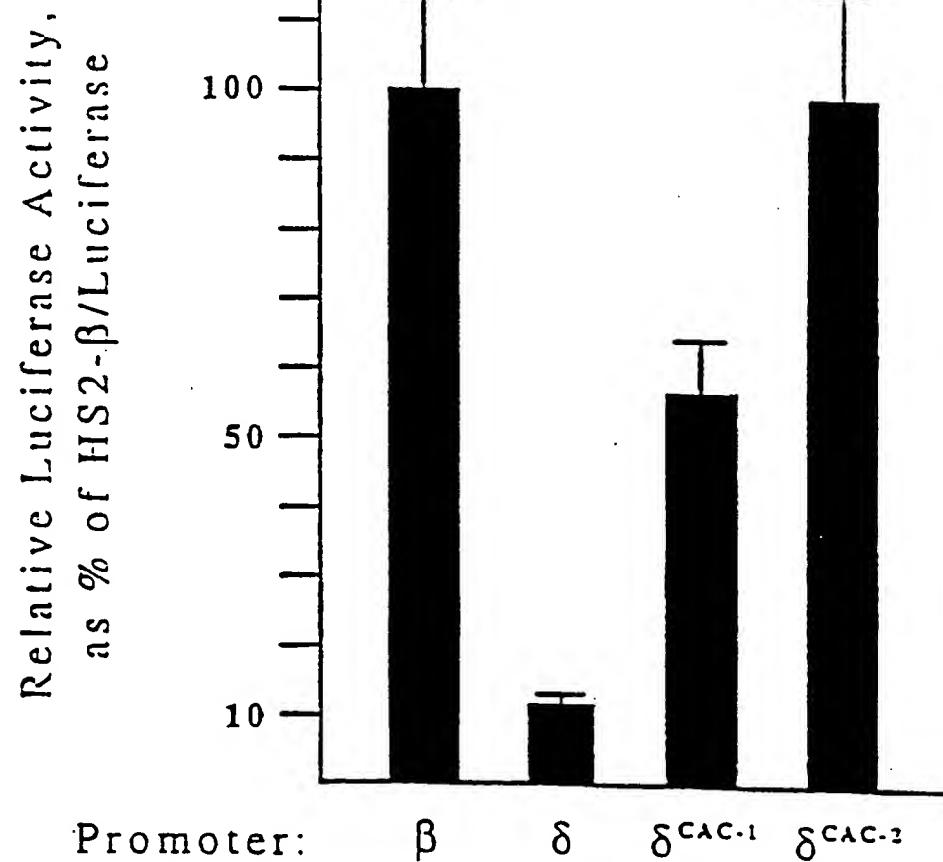
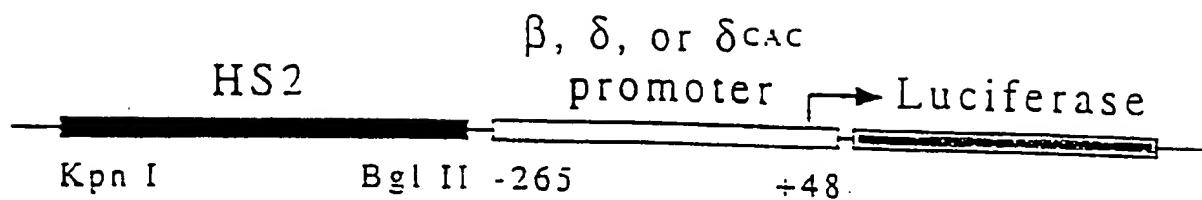


FIG. 3B

FIG. 4A

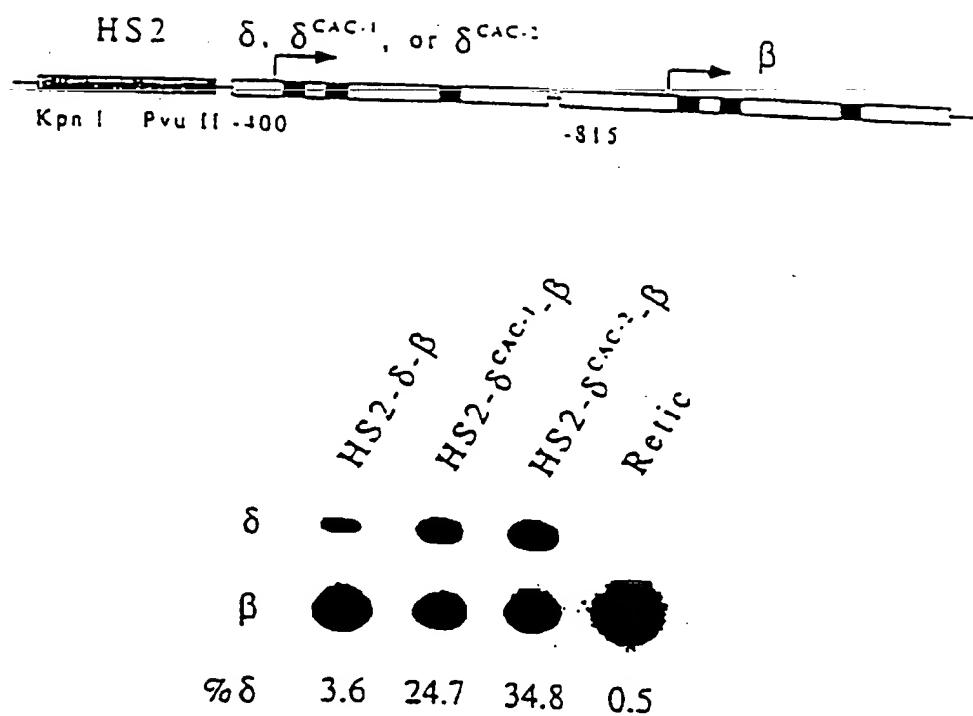


FIG. 4B

FIG. 5A

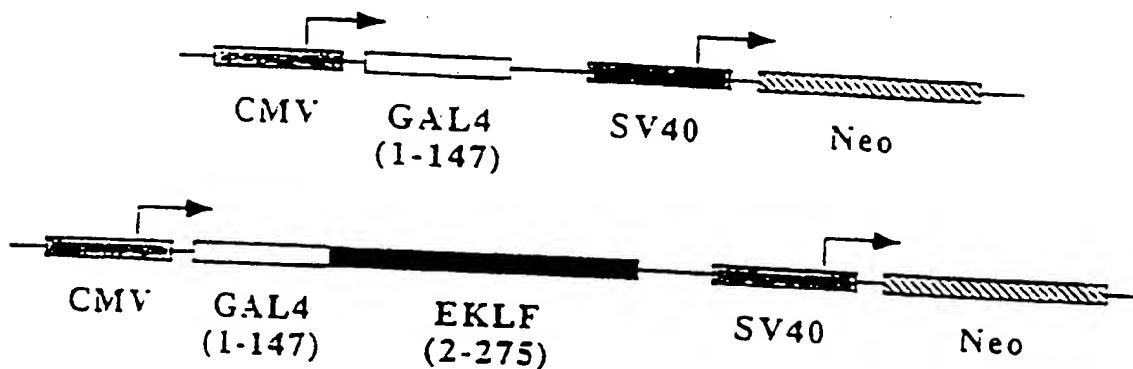
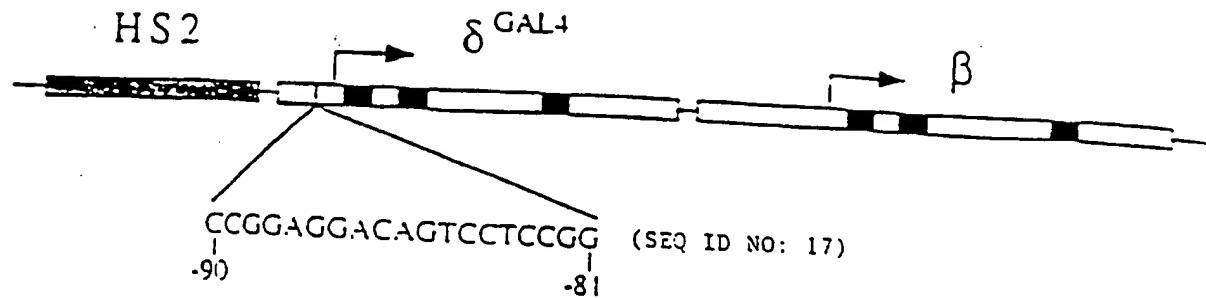


FIG. 5B

$pCH_{neo} \cdot \delta \cdot \beta^*$
 $pCH_{neo} \cdot \delta \cdot GAL4$
 $pCH_{neo} \cdot GAL4 \cdot \beta^*$
 $pCH_{neo} \cdot GAL4 \cdot EKLF$

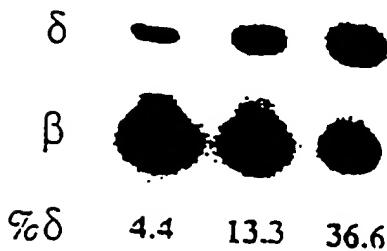
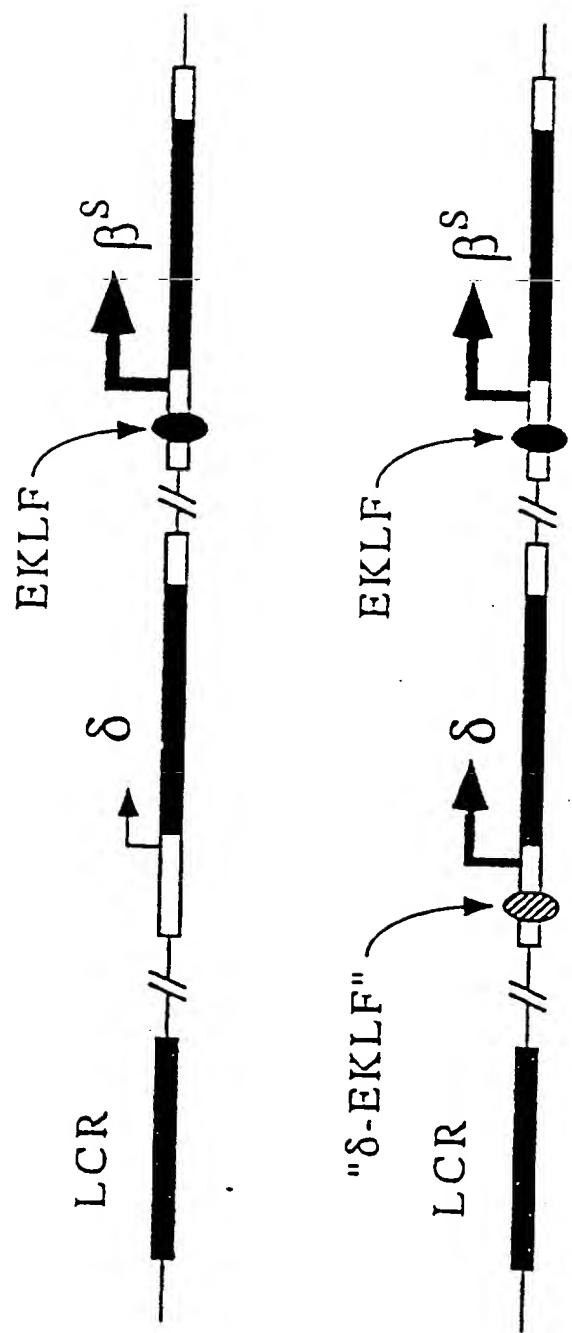


FIG. 5C

FIG. 6



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/10176

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 31/70; C12N 15/63

US CL : 514/44; 435/69.1, 320.1, 325, 91.2, 6; 536/23.5, 24.31; 530/350

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44; 435/69.1, 320.1, 325, 91.2, 6; 536/23.5, 24.31; 530/350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSIS, EMBASE, CAPLUS, WPIDS
search terms: erythroid kruppel like factor, EKLF, delta globin, gene therapy, hemoglobinopathies, thalassaemias

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------|--|-----------------------|
| Y | BIEKER. Isolation, genomic structure, and expression of human erythroid Kruppel-like factor (EKLF). DNA and Cell Biology. 1996, Vol. 15, No. 5, pages 347-352, see entire document. | 1-10 |
| Y | MILLER et al. A novel, erythroid cell-specific murine transcription factor that binds to the CACCC element and is related to the Kruppel family of nuclear proteins. Molecular and Cellular Biology. May 1993, Vol. 13, No. 5, pages 2776-2786, see entire document. | 1-10 |

Further documents are listed in the continuation of Box C. See patent family annex.

| | |
|--|--|
| * Special categories of cited documents: | |
| *A* document defining the general state of the art which is not considered to be of particular relevance | *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
| *E* earlier document published on or after the international filing date | *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
| *L* document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another document or other special reason (as specified) | *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| *O* document referring to an oral disclosure, use, exhibition or other means | &* document member of the same patent family |
| *P* document published prior to the international filing date but later than the priority date claimed | |

Date of the actual completion of the international search

09 SEPTEMBER 1997

Date of mailing of the international search report

02 OCT 1997

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Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US97/10176

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------|---|-----------------------|
| A | GREGORY et al. Functional interaction of GATA1 with erythroid Kruppel-like factor and Spl at defined erythroid promoters. Journal of the American Society of Hematology. 01 March 1996, Vol. 87, No. 5, pages 1793-1801, see entire document. | 1-10 |
| A | PERKINS et al. Lethal beta-thalassaemia in mice lacking the erythroid CACCC-transcription factor EKLF. Nature. 25 May 1996, Vol. 375, pages 318-322, see entire document. | 1-10 |
| A | NUEZ et al. Defective haematopoiesis in fetal liver resulting from inactivation of the EKLF gene. Nature. 25 May 1996, Vol. 375, pages 316-318, see entire document. | 1-10 |
| A | DONZE et al. Role of erythroid Kruppel like factor in human gamma- to beta-globin gene switching. Journal of Biological Chemistry. 27 January 1995, Vol. 270, No. 4, pages 1955-1959, see entire document. | 1-10 |
| A | BIEKER et al. The erythroid Kruppel-like factor transactivation domain is a critical component for cell-specific inducibility of a beta-globin promoter. Molecular and Cellular Biology. February 1995, Vol. 15, No. 2, pages 852-860, see entire document. | 1-10 |
| A | FENG et al. Analyses of beta-thalassemia mutant DNA interactions with erythroid Kruppel-like factor (EKLF), an erythroid cell-specific transcription factor. Journal of Biological Chemistry. 14 January 1994, Vol. 269, No. 2, pages 1493-1500, see entire document. | 1-10 |
| A | MERIKA et al. Functional synergy and physical interactions of the erythroid transcription factor GATA-1 with the Kruppel family proteins Spl and EKLF. Molecular and Cellular Biology. May 1995, Vol. 15, No. 5, pages 2437-2447, see entire document. | 1-10 |
| A, E | TANG et al. Restoration of the CCAAT Box or insertion of the CACCC motif activate delta-globin gene expression. Blood. 01 July 1997, Vol. 90, No. 1, pages 421-427, see entire document. | 1-10 |
| X, P | DONZE et al. Activation of delta-globin gene expression by erythroid Kruppel-like factor: a potential approach for gene therapy of sickle cell disease. 15 November 1996, Vol. 88, No. 10, pages 4051-4057, see entire document. | 1-10 |